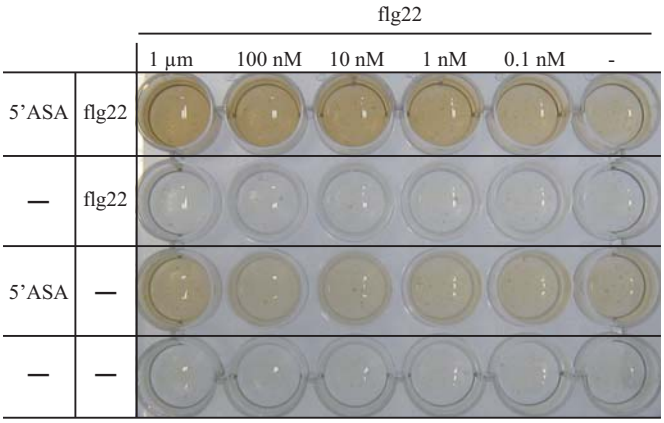
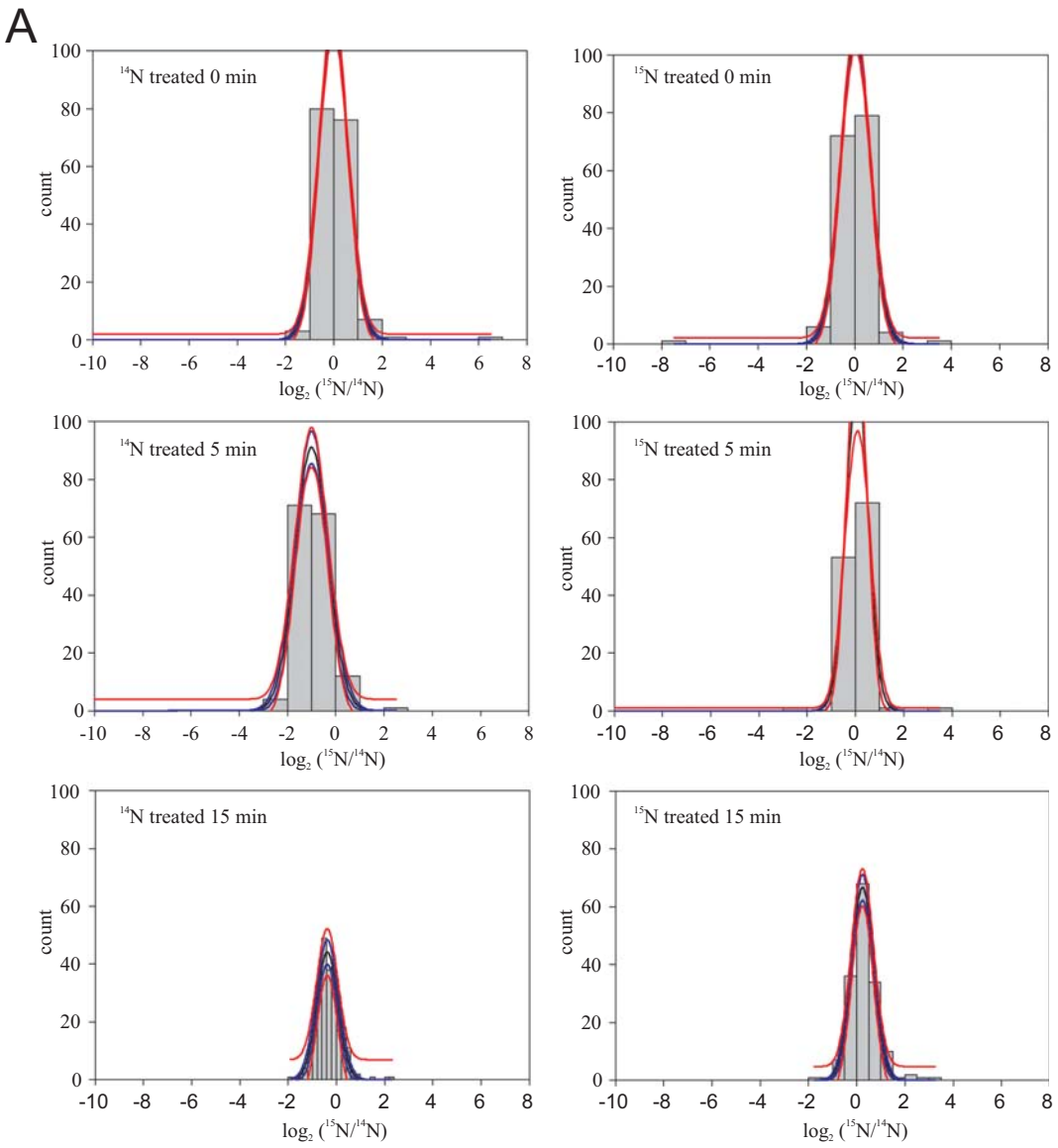
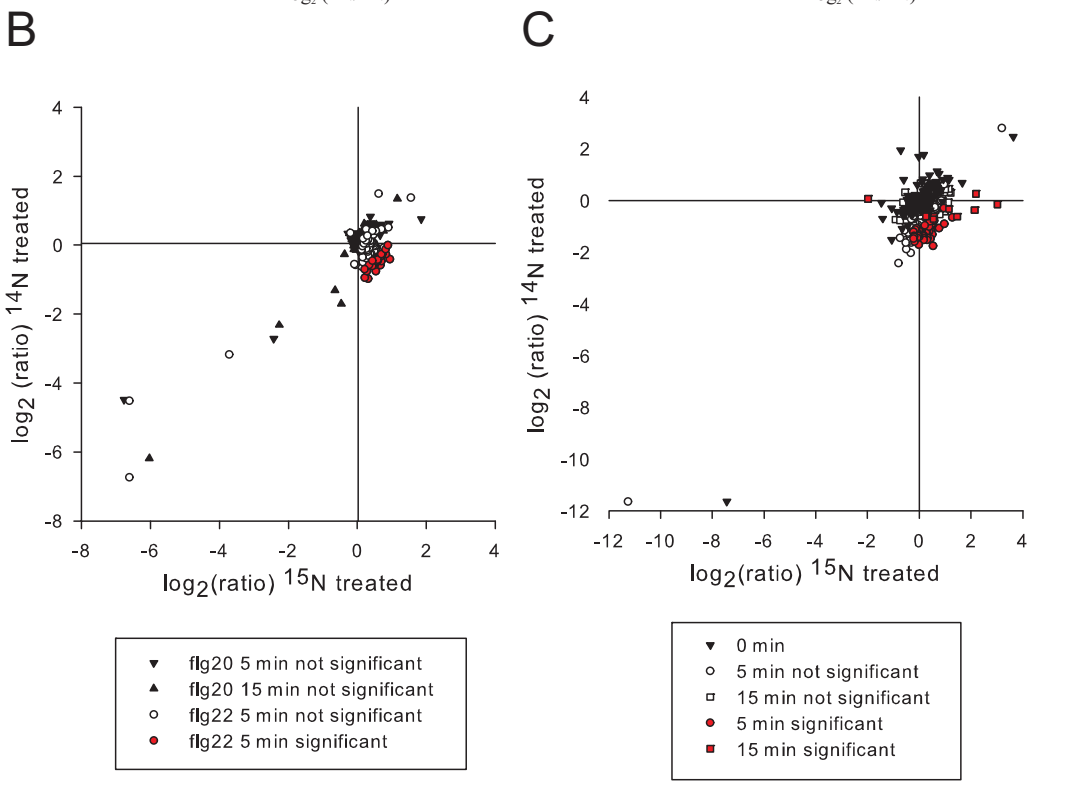

flg22
1 µm
100 nM
10 nM
1 nM
0.1 nM
-
5'ASA
flg22
—
flg22
5'ASA
—
—
—

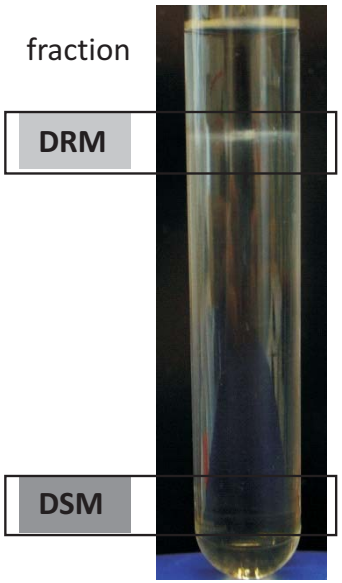

A
^{14}N treated 0 min
^{15}N treated 0 min
^{14}N treated 5 min
^{15}N treated 5 min
^{14}N treated 15 min
^{15}N treated 15 min
count
log$_2$ (^{15}N/^{14}N)

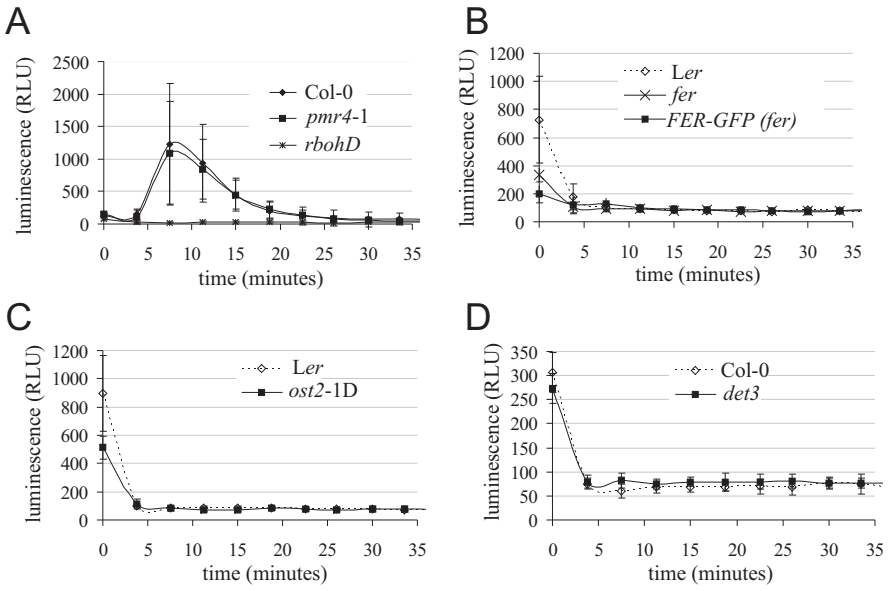

B
log2 (ratio) 14N treated
log2(ratio) 15N treated
flg20 5 min not significant
flg20 15 min not significant
flg22 5 min not significant
flg22 5 min significant
C
log2 (ratio) 14N treated
log2(ratio) 15N treated
0 min
5 min not significant
15 min not significant
5 min significant
15 min significant

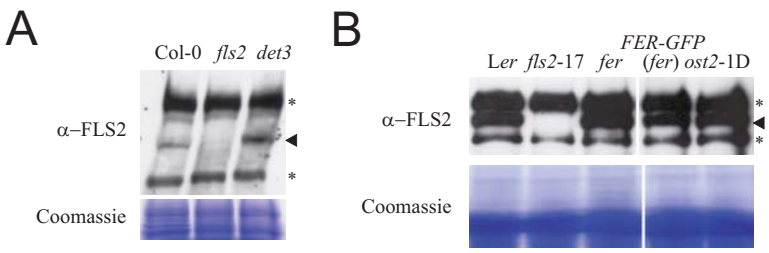
A

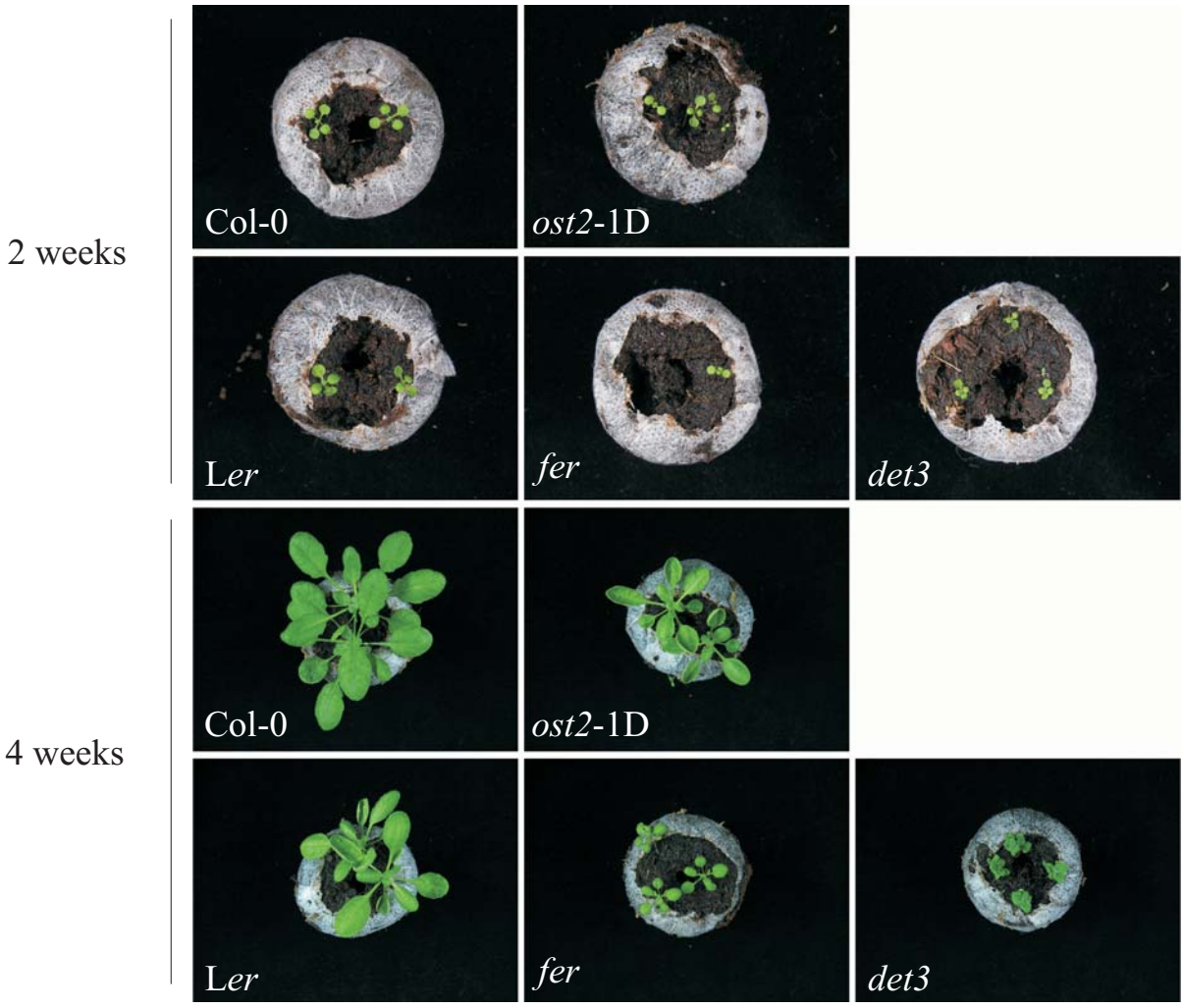
	AGI and description	treatment	average log_2(ratio treated/untreated) flg22 vs flg22Δ2 5 min	flg22 vs flg22Δ2 15 min	flg22 5 min	flg22Δ2 5 min	
Responding proteins	AT1G12840			0,63			DRM
	DET3 (DE-ETIOLATED 3)			-0,18			DSM
	AT1G789000		0,66	0,33			DRM
	VHA-A (VACUOLAR ATP SYNTHASE SUBUNIT A)		-0,40				DSM
	AT3G02880		0,62	0,07	0,37	0,01	DRM
	leucine-rich repeat transmembrane protein kinase		-0,66				DSM
	AT4G12420		0,66	2,21	0,73	0,10	DRM
	SKU5; copper ion binding / oxidoreductase		-0,17		0,13	0,19	DSM
Non-responding proteins	AT2G45470			0,49			DRM
	FLA8 (FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 8)			0,12			DSM
	AT3G01390						DRM
	VMA10 (VACUOLAR MEMBRANE ATPASE 10)				-0,03		DSM
	AT3G07390		0,25	0,38	0,10	0,13	DRM
	AIR12; extracellular matrix structural constituent			0,45	-0,05	0,18	DSM
	AT4G12730		0,11	0,49	-0,06	0,15	DRM
	FLA2 (FASCICLIN-LIKE ARABINOGALACTAN 2)				-0,03		DSM
	AT4G26690		0,09	0,30			DRM
	SHV3 (SHAVEN 3); glycerophosphodiester phosphodiesterase/				0,01		DSM
	AT4G31840		0,02	0,31	0,13	0,07	DRM
	plastocyanin-like domain-containing protein				-0,14		DSM
	AT5G55480		0,17	0,24	0,23		DRM
	SVL1 (SHV3-LIKE 1); glycerophosphodiester phosphodiesterase/				-0,05		DSM

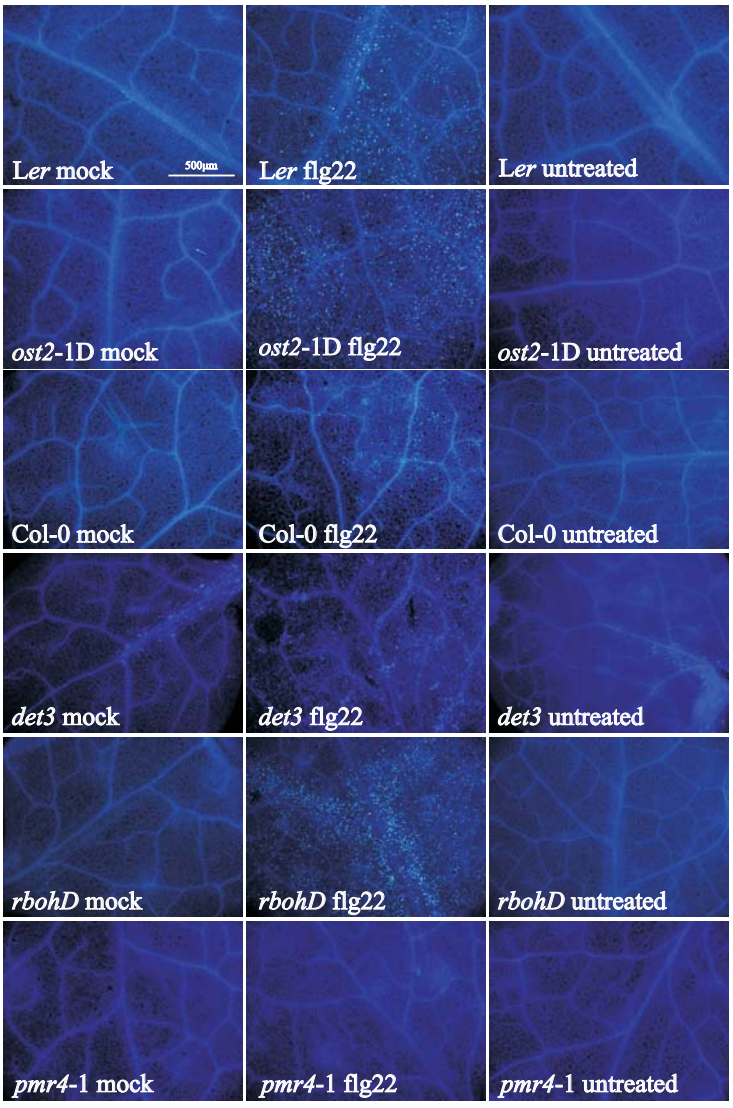
B

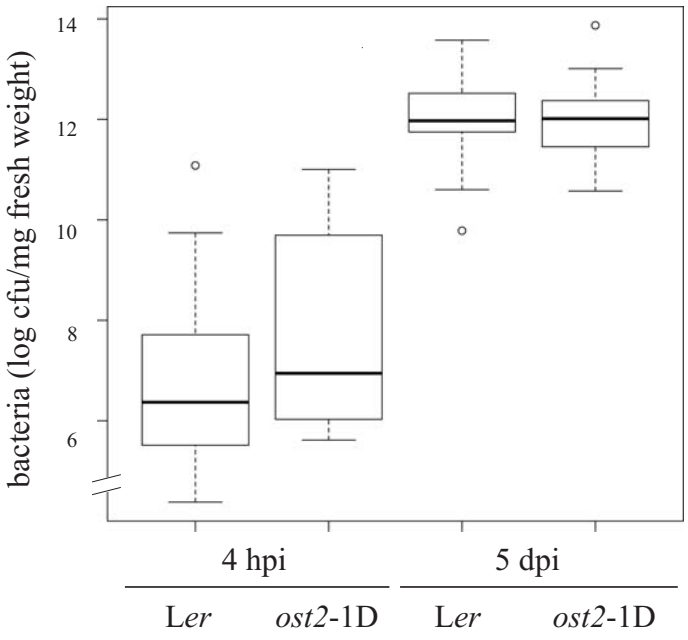



A
luminescence (RLU)
2500
2000
1500
1000
500
0
Col-0
pmr4-1
rbohD
0 5 10 15 20 25 30 35
time (minutes)
B
luminescence (RLU)
1200
1000
800
600
400
200
0
Ler
fer
FER-GFP (fer)
0 5 10 15 20 25 30 35
time (minutes)
C
luminescence (RLU)
1200
1000
800
600
400
200
0
Ler
ost2-1D
0 5 10 15 20 25 30 35
time (minutes)
D
luminescence (RLU)
350
300
250
200
150
100
50
0
Col-0
det3
0 5 10 15 20 25 30 35
time (minutes)


A
Col-0
fls2
det3
α–FLS2
Coomassie
B
FER-GFP
Ler
fls2-17
fer
(fer) ost2-1D
α–FLS2
Coomassie


2 weeks
4 weeks
Col-0
ost2-1D
Ler
fer
det3
Col-0
ost2-1D
Ler
fer
det3


Ler mock
500µm
Ler flg22
Ler untreated
ost2-1D mock
ost2-1D flg22
ost2-1D untreated
Col-0 mock
Col-0 flg22
Col-0 untreated
det3 mock
det3 flg22
det3 untreated
rbohD mock
rbohD flg22
rbohD untreated
pmr4-1 mock
pmr4-1 flg22
pmr4-1 untreated


bacteria (log cfu/mg fresh weight)
14
12
10
8
6
4 hpi
5 dpi
Ler
ost2-1D
Ler
ost2-1D

SUPPLEMENTAL FIGURE LEGENDS

Figure S1
Arabidopsis cell culture is responsive to flg22. *Arabidopsis* cell culture was distributed in 1 ml aliquots into culture plates and supplied with the peroxidase substrate 5' aminosalicylic acid (5'ASA) as well as indicated concentrations of flg22 to monitor the oxidative burst. As negative controls, cells were treated with 5'ASA or flg22 only or cells were left untreated. Cell culture plates were analyzed visually 1.5 h after induction. The experiment was performed twice with similar results.

Figure S2
Histograms of $^{15}N/^{14}N$ ratios and response of proteins to treatment with flg22 or flg22Δ2. (A) Exemplarily shown is the frequency distribution of log_2-transformed ratios of the ^{15}N to ^{14}N form of proteins from one of the label-swap experiments (flg22 vs. untreated). In this experiment, cells were either treated with active flg22 or left untreated (see Fig. 1B). In all samples, the observed label ratios show a normal distribution centered around zero and match Gaussian curves (blue line, Gauss curves fitted on the histograms; red lines, 95% confidence interval). The width of the distributions broadens with time indicating more responsive proteins at later time points during flg22 treatment. (B and C) Log_2 values of ^{15}N to ^{14}N ratios from one experiment were plotted against log_2 values of ^{15}N to ^{14}N ratios from the reciprocal experiment. (B) Summary of the results for treatment with flg22 compared to untreated cells, as well as for flg22Δ2 compared to untreated cells (see Fig. 1B). (C) Summary of the results for an experiment of one cell type treated with flg22 as the treatment and the other cell type was treated with flg22Δ2 as control (see Fig. 1A). Red symbols indicate those proteins which show significant reciprocal response based on the procedure described in SI Methods (1).

Figure S3
Ratios of protein abundance in the detergent-resistant membrane fraction (DRM) or the detergent-soluble fraction (DSM) of flg22-treated versus flg22Δ2-treated/untreated cells as determined by $^{15}N/^{14}N$ ratiometric proteomic analysis. (A) For the proteins identified as flg22-responsive in Table S1, the increase in abundance in the DRM fraction (indicated by positive values) is accompanied by a tendency towards a decrease in the DSM fraction (indicated by negative values). For proteins considered as non-responding in Table S1, essentially unaltered protein abundance in both DRM as well as DSM fractions was observed. (B) Distribution of the DRM and DSM fractions within the sucrose gradient in an ultracentrifugation tube following centrifugation at 250,000 x *g* for 18 h.

Figure S4
The *pmr4*-1 mutant displays an oxidative burst similar to wild-type, and *det3*, *fer* and *ost2*-1D do not show ROS production without addition of flg22. ROS production in response to 100 nM flg22 in *pmr4*-1 (A), *rbohD* (A), *fer* (B), *ost2*-1D (C) and *det3* (D) as well as the respective wild-type was measured indirectly as relative light units (RLU). Error bars represent standard deviation of six to twelve (A), twelve (B), six (C) or 14 (D) independent samples measured in a single experiment. The experiment was performed five (A), two (B-D) times with similar results.

Figure S5
FLS2 immunoblot analysis. (A and B) *det3*, *fer*, *FER-GFP*(*fer)* and *ost2*-1D accumulate wild-type-like FLS2 levels. Total protein was extracted from seedlings and samples were analyzed by immunoblot analysis using α-FLS2 antibody (upper panels). Equal loading is indicated by Coomassie staining (lower panels). (A) and (B) are derived from separate protein extractions. (B) All displayed lanes are derived from the same blot; one band harboring an unrelated sample was excised as indicated by the white space. Arrowheads indicate FLS2 and asterisks unspecific bands.

Figure S6
Juvenile and adult growth phenotype of *det3*, *fer* and *ost2*-1D. Plants were grown on Jiffy pellets and photographs taken at the time points indicated.

Figure S7
det3, *ost2*-1D and *rbohD* retain flg22-induced callose deposition. *det3*, *ost2*-1D, *rbohD*, *pmr4*-1 as well as respective wild-type plants were treated with 2 μM flg22 and callose deposition was visualized by aniline blue staining. Exemplary micrographs (derived from different experiments) of rosette leaves either mock-treated or 24 h after addition of flg22 are shown. Bar = 500 μm.

Figure S8
ost2-1D shows unaltered susceptibility to PtoDC3000Δ*avrPto*Δ*avrPtoB*. *Arabidopsis ost2*-1D and respective wild-type plants were inoculated with PtoDC3000Δ*avrPto*Δ*avrPtoB* by surface inoculation. Bacteria were extracted from surface-sterilized leaves at 4 hours post inoculation and 5 days post inoculation. Depicted is a box-plot diagram representing the statistical distribution of the data. Thick lines indicate the median, boxes represent the interquartile range, whiskers indicate the whole data range and dots represent outliers. The experiment was performed three times, the box plot includes all three data sets.

SUPPLEMENTAL TABLE HEADINGS

Table S1
Compilation of quantified proteins.

Worksheet 1 (summary): For all proteins present in both samples of a reciprocal pair quantitation was pursued. Proteins significantly enriched in DRMs after flg22 treatment are indicated in bold ($p < 0.05$). Functional category (FC); *Arabidopsis* Genome Initiative code (AGI code); distance (D); maximal fold-change (max fold); average fold-change (av fold); probability-value (p); number of TM domains based on the consensus predicted by ARAMEMNON (TM, (2)); experimental evidence for PM association (PM, (2-5)); transcriptionally co-expressed with *FLS2* (6), number indicates rank of co-expressed gene according to ATTED (ATTED); elevated transcript levels in response to flg22 treatment (flg22 up, (7,8)); phosphorylated after flg22 treatment (P flg22, (9,10)); (putative) mutants of according genes were analyzed for flg22 responsiveness in this study (MA); flg22-induced reactive oxygen species production (ROS). enriched (enr.), dephosphorylated (de-p), phosphorylation below the significance threshold (✓), not germinated (ng), no ROS = 1, weak ROS = 2, wild-type ROS = 3, ROS higher than wild-type = 4.

Footnote worksheet 1:
[A]PMR4 required for wound and papillary callose formation (11,12).
[B]*NHL3* (*NDR1/HIN1-LIKE*) transcript accumulation was specifically observed during the interaction with avirulent *Pseudomonas syringae* strains (13). NHL3-overexpressing plants are more resistant to *Pseudomonas syringae* (14). Interestingly, NDR1, one of the founders of the *NDR1*/*HIN1*-like gene family, was shown to interact with RPM1 INTERACTING PROTEIN4 (RIN4), a negative regulator of plant immunity (15).
[C]Group 11b REMORINS have been observed to be differentially expressed during *Arabidopsis*-*Pseudomonas syringae* interactions (16). *Potato virus X* (PVX) movement is inversely related to REMORIN accumulation in transgenic tomato plants (17).

Worksheet 2 (all peptides of 15N-14N): Full list of all proteins and respective peptide identifications in all $^{15}N/^{14}N$-label experiments. For each peptide, the experimental m/z value, charge state, Mascot score and mass accuracy is listed. Quantitative values (^{15}N to ^{14}N ratio and standard deviation) are listed for each peptide.

Worksheet 3 (ion intensities of each peptide): Unlabeled cells were either treated with flg22 or remained untreated, and samples were taken at 0, 5 and 15 minuntes. Ion intensities of each peptide m/z value were normalized to the total sum of ion intensities within each sample. Subsequently ratios of treated to untreated normalized intensities were calculated for each time point.

Worksheet 4 (label-free quantification): Ratios of mean ion intensities (see worksheet 3) of proteins from treated or untreated cell extracts. Peptide ratios were averaged to obtain protein ratios. Ratios of

two replica sets were averaged and standard deviation were calculated. The inserted histogram shows Gaussian distribution of log_2-transformed ratios of ion intensities (treated/untreated cells at 5 and 15 minuntes). The width of the distribution broadens with time indicating more responsive proteins at the later time point during flg22 treatment.

SUPPLEMENTAL METHODS

Plant material and growth conditions

Arabidopsis thaliana (Col-0) cell cultures derived from leaves were grown under continuous light (80 to 100 $m^{-2}s^{-1}$) at 24°C in JPL medium with 10 mM potassium nitrate as sole nitrogen source (18). *det3* (19), *ost2*-1D (20), *fer* (genotype *fer/fer;pp2c/pp2c*), *FER-GFP*(*fer*) (genotype *fer/fer;pp2c/pp2c;pFER::FER-GFP*) as well as the respective wild-type plants were grown on soil or Jiffy pellets (Jiffy, Lorain, OH) for approximately four weeks at a day/night cycle of 10:14 h, with 22°C:20°C day/night temperature and a relative humidity of 60%. T-DNA mutants (Table S1; (21,22)) were obtained from the *Arabidopsis* stock centre (http://arabidopsis.info/). A homozygous *fer/fer* mutant was identified in the progeny of heterozygous *Fer/fer* plants (23). *fls2*-17 (24), *fls2* (8), *pmr4*-1 (12) and *rbohD* (25) as well as the respective wild-type plants were grown on soil for approximately four weeks at a day/night cycle of 10:14 hrs, with 22°C:20°C day/night temperature and a relative humidity of 60%. All genotypes prescreened with the oxidative burst assay are listed in Table S1 (21,22). T-DNA insertion lines (53 out of 57 annotated as homozygous) obtained from the SALK collection were not verified by PCR.

Oxidative burst assay for cell culture

Oxidative burst assays were performed as previously described (26). Briefly, cell culture suspensions were distributed in 1 ml aliquots into culture plates and supplied with the peroxidase substrate 5' aminosalicylic acid (5'ASA, 400 µM). Then flg22 was added to the indicated final concentrations.

Oxidative burst assay for seedlings

Oxidative burst assays for seedlings were essentially performed as described for leaf discs (26). Briefly, plants were germinated on solid MS medium containing 1% sucrose supplemented with Nitch vitamins (MSN) and at ten days transferred to liquid MSN medium or germinated directly in liquid MSN medium. At fourteen days seedlings were pre-stimulated with flg22 (final concentration 10 nM for 1 h) followed by 1 h incubation in water. Finally, the reaction mixture, containing horseradish peroxidase (20 µg, Fluka), luminol (400 µM, Fluka) and flg22 (100 nM final concentration), was added. Seedlings treated with ConcanamycinA (Sigma-Aldrich) were preincubated with ConcanamycinA (1 mM stock in dimethyl sulfoxide, 5 µM final concentration) for 2 h. Control samples were treated with the respective amount of dimethyl sulfoxide.

Analysis of callose deposition (aniline blue staining)

To assess flg22-induced callose deposition, seedlings were treated and stained as described previously (27). Briefly, 2-week-old seedlings grown under sterile conditions in 48-well plates containing solid MSN (1% sucrose, 1% agar) were overlaid with 2 µM flg22 in MSN and harvested 24 hrs later. Seedlings were cleared with ethanol:acetic acid (1:3 (v/v)), subsequently stained for 24 hrs with 0.01% aniline blue in 150 mM KH_2PO_4 (pH 9.5) and callose was visualized in true leaves by epifluorescence microscopy.

Analysis of cell death (trypan blue staining)

$MgCl_2$ was syring-infiltrated into rosette leaves of 4 week-old plants. Samples were harvested at 24 h after infiltration and boiled shortly in Trypan blue solution (0.1 mg/ml) (28). Subsequently the leaves were destained in chloral hydrate (2.5 mg/ml) and analyzed by light microscopy to visualize dead cells.

MAPK activity assay

MAPK activity was visualized in two week-old seedlings by immunodetection of phosphorylated MAPKs using a polyclonal phospho-p44/42 MAPK-specific antiserum (Cell Signaling Technology, Danvers, MA). Seedlings were grown for 10 days on solid MSN (1% sucrose, 1% agar) medium and

then transferred to liquid medium of the same composition. After four days, the liquid medium was replaced by either water (0 min) or 100 mM flg22. Samples were either taken immediately (0 min) or at 15 and 30 min, respectively. Crude extracts were prepared in phosphatase-inhibiting buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 25 mM β-glyero-phosphate, 2 mM sodium orthovanadate, 10% glycerol, 0.1% Tween, 1 mM DTT, 1mM PMSF and one complete protease inhibitor tablet (Roche Applied Science, Indianapolis, IN) per 50 ml extraction buffer) by homogenizing tissue in a BeadBeater device. Cell debris was removed by centrifugation and protein samples (20 µg each) boiled for 10 min in 5x loading buffer. Following gel electrophoresis, proteins were blotted onto nitrocellulose membranes and the transfer visualized with the Ponceau protein dye. Subsequently blots were first incubated with the phosphor MAPK antiserum (1:2000 dilution in PBST at 4°C over night) and then with a horseradish peroxidase-coupled goat anti rabbit secondary antiserum (Santa Cruz, Biotechnology, Santa Cruz, CA; 1:5000 dilution in PBST). For signal detection SuperSignal West Pico and SuperSignal West Femto solutions (Pierce, Fisher Scientific, Pittsburgh, PA) were used as a 9+1 mixture. Luminescence was documented on X-ray films.

Measurement of stomatal aperture

Quantification of stomatal aperture was essentially performed as described in (29). Seedlings were grown for one week on solid MSN medium (1% sucrose, 1% agar) and then transferred to liquid MSN medium. At the age of two weeks, seedlings with fully opened stomata (being at least since three hours in light conditions) were vacuum-infiltrated with water (with or without flg22; 3 µM final concentration) and incubated for two hours at room temperature. First true leaves were analyzed by light microscopy, and stomatal aperture (width and length) quantified on the basis of micrographs using ImageJ software (http://rsbweb.nih.gov/ij/). Six leaves from six different seedlings were analyzed per genotype and experiment.

***Pseudomonas syringae* infection assay**

Bacterial infections and statistical analysis were performed as previously described (30). Briefly, two-week old soil-grown seedlings were sprayed with *Pto*DC3000Δ*avrPto*Δ*avrPtoB* at 0.5 x 10^8 cfu/ml. Whole seedlings (root cut) were sampled at 4 hpi and 5 dpi and surface sterilized for 20 s in 70% ethanol. A total of 10 seedlings (two per sample; five samples per genotype and time point) were weighed, bacteria extracted and several dilutions plated on medium containing appropriate antibiotics. Results of three independent experiments were combined and statistical analysis (ANOVA and subsequent post-hoc test by Tukey's HSD) was done using R software (www.r-project.org).

Protein extraction and immunoblotting (FLS2 Western blot of total protein)

Total proteins were isolated from ground two week-old seedlings using extraction buffer (20 mM Hepes pH 7.5, 13% (w/v) sucrose, 1 mM EDTA) containing protease inhibitor cocktail (Roche), 1 mM DTT and 1% Triton X-100. Tissue debris was spun down and samples were boiled for 10 min in 2x loading buffer (125 mM Tris pH 6.8, 25% (v/v) glycerol, 5% (v/v) SDS, 0.1% (w/v) Bromophenol Blue, 200 mM DTT). Samples were analyzed by immunoblot analysis using α-FLS2 antibody (31).

FLS2 Western blot (PM-derived DRMs)

PM-derived DRMs were prepared from cultured cells as described above. After SDS-polyacrylamide gel electrophoresis using a Tris-Glycine gradient gel 4% - 12% (NuPAGE; Invitrogen, Carlsbad, CA), proteins were blotted onto PVDF membrane (Imobilon; Millipore, Billercia, MA) using semi-dry protein transfer (Trans-Blot SD; Biorad, Hercules, CA). The membrane was blocked in 5% (w/v) milk TBS-T (140 mM NaCl, 2.5 mM KCl, 25mM Tris-HCl pH 7.4, 0.05% Tween20) over night at 4°C. The primary α-FLS2 antiserum (18) was added to the blocking solution at a concentration of 1:5000 and incubated at room temperature for at least 1 hour under constant shaking. The secondary alkaline phosphatase-coupled antibody (goat anti-rabbit) was used at a concentration of 1:30000 for 1 hour at room temperature. For detection, blots were incubated with chemi-luminescence detection solution (CDP-STAR; Perkin Elmer, Waltham, MA) and light emission was documented on X-ray films. For total protein detection on PVDF membranes after electroblotting, membranes were stained with Coomassie in 0.1 % (w/v) Coomassie Blue R-250 in 50% (v/v) methanol for 15 minutes and destained in 50% (v/v) methanol, 10% (v/v) acetic acid until protein bands were visible. Background staining was further reduced by washing with water.

Allocation of functional categories

Protein functional categories were assigned according to MapMan (32). Categorization was adjusted manually for obviously wrongly annotated proteins/genes (At1g32050, At1g05570, At2g45820, At3g61260, At4g04720, At2g36910, At4g29900, At3g13380, At3g51740, At1g53100, At3g13560, At5g42100, At3g58100, At4g35230, At2g47060, At3g17410, At4g03550; AT1g11330, At5g13000, At3g07160, At1g64760, At3g13560, At5g42100, At3g58100, At5g56590, At4g31140 and At5g58090).

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